DEPARTMENT OF HEALTH AND HUMAN SERVICES

NOTE TO FILE (BNF32)

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Subject: Male sterile and fertility restorer oilseed rape lines

Keywords:
Oilseed rape, *Brassica napus*, male sterile, fertility restorer, *Escherichia coli*, *neo* (*kanR*), kanamycin resistance, *Streptomyces hygroscopicus*, *bar*, phosphinothricin acetyltransferase (PAT), herbicide tolerant, *Bacillus amyloliquefaciens*, *barnase*, RNase, *barstar*, RNase inhibitor, pollination control system.

Background

In a primary submission dated 6 July 1995, Plant Genetic Systems N.V. (PGS) provided summary information to support their safety and nutritional assessments of their new oilseed rape (*Brassica napus*) lines, engineered for development of a novel pollination control system. These lines contain transformation events B91-4 (male sterility) and B93-101 (fertility restoration). In a second submission dated 23 October 1995, PGS provided information for another fertility restoration line containing transformation event B94-2. Additional information was received on 14 February, 23 February and 4 March 1996.

Intended Effect and Food/Feed Use

Oilseed rape ia a crop capable of both self-pollination (70%) and cross-pollination (30%); hence, a pollination control system is required to produce 100% F_1 hybrid seed. PGS reports the development of a novel genetically engineered hybridization system applicable to many crop types, and specifically reports its introduction in oilseed rape in the above submissions. In their system, the male sterile line prevents self-pollination, which allows the production of 100% true hybrid seed. Fertility restoration, then, ensures that hybrid seed is itself fertile in the growing field.

The PGS pollination control system is designed to overcome the disadvantages associated with cytoplasmic male sterility, which include: unreliability; undesirable side effects in male sterile plants; instability of the sterility trait; and the lack of a linked marker in most male sterile mutations, thereby precluding use in F_1 rape production. Experimental F_1 hybrid progeny, resulting from cross-pollination or hybridization, typically produce better yields, are more uniform, and are more adaptable to different

environmental conditions than either parent. Therefore, the intended technical effect of the aforementioned genetic modifications is the development of a new hybridization system "based on a dominant nuclear male sterility (NMS) gene that is linked to a convenient marker, and a restorer of fertility gene linked to the same marker gene."

F_1 hybrids of oilseed rape varieties have 20-25% greater yields than optimal open-pollinated oilseed rape varieties. Greater hybrid uniformity also facilitates harvesting and marketing. "The PGS hybridization system enables seed production of 100% hybrid populations; a quality not achieved by any other hybridization systems applied in oilseed rape."

Oilseed rape is grown for its seed, which, in turn, is further processed to yield oil and meal for human food and animal feed, respectively. Nectar, the raw material for honey production, is also produced from the plant. Classical breeding has allowed the commercial development of crop varieties, by eliminating and/or lowering the levels of the anti-nutritional factors erucic acid and glucosinolates. These new varieties have previously been the subject of regulatory consultations as canola, which includes the oil and meal derived from them. Strict standards have been accepted for canola oil and canola meal.

Molecular Alterations and Characterization

Cointegrative disarmed plasmids were constructed by PGS to transfer the DNA sequences of interest to oilseed rape. The *Agrobacterium*-mediated plant transformation system (simple integration), with appropriate regulatory elements (specific plant promoters and 3' end polyadenylation signal sequences), was used to incorporate T-DNA from the plasmids pTTM8RE and pTVE74RE, which were engineered to encode male sterility (B91-4) and restoration of fertility (B93-101 and B94-2), respectively.

The functional genes present in the constructs were isolated from bacteria and, as described by PGS, include: 1. The *neo* (*kanR*) antibiotic resistance gene, isolated from a *Tn5* carrying plasmid of *Escherichia coli*, encoding the enzyme neomycin phosphotransferase II (APH(3')II) (NPTII) and used for in vitro selection of transformed plant cells; 2. The *bar* (*pat*) gene, isolated from *Streptomyces hygroscopicus*, encoding phosphinothricin acetyl transferase (PAT), and used for efficient field selection of modified plants by tolerance to phosphinothricin herbicide; 3. The *barnase* and *barstar* genes, isolated from *Bacillus amyloliquefaciens*, encoding two small single-chain proteins designated barnase and barstar, respectively. Barnase is a specific extracellular ribonuclease (RNase) which disrupts normal cell functions. Barnase expression is confined to the tapetum cell layer

of anthers (by virtue of the PTA29 promoter) and its expression arrests early anther development leading to male sterility. Barstar protein is a specific barnase inhibitor which when co-expressed in rape with barnase prevents male sterility. Barstar forms a one-to-one complex with barnase, inactivating the RNase. Barstar expressed alone has no effect on plant phenotype.

Expressed Protein

According to PGS, hybridizing the fertility restorer lines with male sterile rape results in co-expression of the *barnase* and *barstar* genes in the tapetum of the F_1 progeny. The tapetum cell layer of the hybrid plant will develop normally making the plants 100% fertile. For crops which are primarily self-pollinated and where seed (or fruit) are harvested from hybrid plants, fertility must be restored in the hybrid offspring to assure maximal yields.

In the transformed oilseed rape cells, PGS observed that the *neo* and *bar* genes are closely linked to both the chimeric *barnase* and *barstar* genes. Expression of the chimeric *neo* gene provides antibiotic resistance to the aminoglycoside antibiotics (kanamycin, neomycin, geneticin). PGS reported that the genetically altered rape plants may be resistant to up to a few mg kanamycin per ml.

In summary, PGS states that the genetic basis of the hybridization system in the transgenic male sterile B91-4 line and fertility restorer B93-101 and B94-2 lines is as follows:

- the transgenes are inserted as a single copy in a single locus in each line;
- there are no indications of rearrangement of the inserted DNA in lines B91-4 and B93-101; some rearrangement may have occurred in B94-2;
- only the T-DNA between the border sequences has been integrated into the plant genome;
- there is no indication that the insertions occurred in native plant genes;
- each transformant can be identified by a typical molecular integration pattern of the transgenes;
- expression is limited to the intended genes; the transgenes are only expressed in specific oilseed rape organs; there are no indications that the NPTII, PAT, barnase or barstar proteins are present in the transgenic oilseed rape seeds from which oil and cake (meal) may be derived.

Furthermore, PGS reported that the genetic and phenotypic stabilities of the transgenic lines have been demonstrated using standard methods, and that the agronomic performance of the hybridization system has been successfully evaluated in the field.

Allergenic and Toxic Potential

PGS states that oil derived from oilseed rape is the only product consumed by humans and that fats, in general, are rarely allergens.

Additionally, the NPTII, PAT, barnase, and barstar proteins were screened for sequence homology to other known or potential allergens, using the HIVAA7, PIR42 and Swiss-Prot30 databases, and are reported to be unlikely allergens. PGS also reports that employees working closely with the transgenic crop, either in the greenhouse or in the field, have never shown allergic responses or indications thereof in periodic medical examinations.

PGS states that none of the expressed transgenic proteins have been associated with any pathogenic reaction towards humans or animals.

Nutritional Assessment

Oilseed rape oil and cake (meal) are the principle products that enter the human food and animal feed chains. According to PGS, barnase or barstar proteins were not detected in transgenic rape seed, and no messenger RNA (mRNA) was detected coding for these two proteins by Northern blot analyses. The directed expression of PAT suggested its absence in seed, and no *bar* mRNA was detected by Northern blot analyses. Additionally, no *neo* mRNA was detected in transgenic seed. Moreover, no mRNA from any of the inserted genes was detected in transgenic pollen.

PGS further states that oil processing removes protein; hence, any transgenic protein (enzyme) would be eliminated.

A rabbit digestibility experiment using whole rape seeds revealed no adverse effects.

PGS also reported several chemical analyses conducted on rape seed, which included oil and protein contents, fatty acid composition, amino acid profile, and glucosinolate content; overall composition was reported to be comparable between transgenic and control oilseed rape varieties. The firm also indicated that herbicide application did not affect seed quality.

It should be noted that in one instance levels of glucosinolates in the transgenic varieties exceeded the regulatory maximum of 30 umoles/g oil free solid for canola meal. However, these levels of glucosinolates would not preclude sale of the transgenic meal as rapeseed meal.

Conclusions

PGS has concluded that oilseed rape lines containing transformation events B91-4, B93-101, and B94-2 are substantially equivalent to oilseed rape currently grown, marketed, and consumed for animal feed and human food. At this time, based on PGS's description of its data and analyses, the Agency considers PGS's consultation on oilseed rape oil and meal derived from rape lines containing transformation events B91-4, B93-101, and B94-2 to be complete.

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